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October 1949 AIC-254

FERMENTATION PROCESS FOR PRODUCTION OF VITAMIN B_{12}

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The microorganism was isolated in the course of the following screening program. Soil, fecal, or other materials were suitably diluted with sterile water to give 10 to 30 surface colonies on each of a series of agar plates. So that cultures able to grow on chemically simple media would be selected, the following medium was used: Sucrose, 1 percent; citric acid, 0.12 percent; $(NH_4)_2HPO_4$, 0.04 percent; Na_2SO_4, 0.04 percent; KCl, 0.008 percent; Mg, 50 ppm.; Mn, 15 ppm.; Fe, 10 ppm.; Zn, 10 ppm.; Co, 1 ppm.; agar, 1.5 percent; pH 7. The chlorides were used, with the exception of cases otherwise indicated. The plates were incubated at 30°C. for 24 hrs. They were opened and allowed to dry several hours in a warm (50°C.) dry chamber to minimize spreading of moist colonies in the next operation. Each plate was then overlayered with 10 ml. of vitamin B_{12} assay medium containing 1.5 percent agar and inoculated with a suspension of the test organism, L. leichmannii. The plates were then allowed to incubate at 35°C. for 18 to 24 hours. Colonies from which vitamin B_{12} (or compounds with similar activity for L. leichmannii) diffuse are recognized by a halo of growth of L. leichmannii. Skill is necessary, since the halo is very faint in comparison with the vitamin B_{12}-excreting colony. Inspection under a dissecting microscope may aid in acquiring this skill. Representative colonies exhibiting halos large with respect to the size of the colony were picked for further screening. The plate screening method is similar to that described recently by Kelner (Jour. Bact. 56, 158, 1948).

The stocks isolated by the plate-screening method were grown at 25°C. in shake flasks on the sucrose-citrate-salts medium (without agar and with the sucrose concentration increased to 5 percent) mentioned above. Aliquots of whole culture were assayed after maximum growth was obtained, by the conventional L. leichmannii assay. B. megatherium (B-938) gave yields of 150 to 200 millimicrograms of vitamin B_{12} per ml. of whole culture after 4 to 6 days of incubation under these conditions. It was among the more potent isolates at this stage of screening. The more potent isolates were then grown in aerated vats (as described later) in 10 liters of sucrose-citrate-salts medium. Occasionally, supplements such as trypsin-digested casein (N-Z-Amine) or yeast extract were added. The fermentations were allowed to proceed overnight without attention. The whole culture was then concentrated to one-tenth the original volume and the resulting sirup was submitted for chick growth tests. B. megatherium (B-938) proved the most potent in chick tests of approximately 15 cultures submitted. It was isolated from a soil sample from an abandoned chicken yard near Pullman, Washington, obtained through the courtesy of Dr. McGinnis.

Fermentation

B. megatherium (B-938) has given the highest vitamin B_{12} assays (by L. leichmannii) under the following conditions: Fermentor: The laboratory fermentor described by Humfeld (Jour. Bact. 54, 689, 1947) was used. It is advantageous for laboratory investigations, because foam is positively controlled. The fermentor may be allowed to operate for 24-hour periods or longer without attention when expedient. The effect of anti-foam agents on the fermentation may then be investigated as an isolated factor. No anti-foam was used in these studies. A 10-liter volume of medium and an aeration rate of 1 volume of air per volume of medium per minute are used. The fermenter is placed in a bath through which tap water flows, but because of the rate of fermentation the temperature in the medium may rise 10 to 15° above the bath temperature during the period of most rapid growth. The fermentation is started at 30°C., and later is held at about 35°C. by control of the bath temperature.

Culture Medium: The following semi-synthetic medium is used: sucrose, 5 percent; yeast extract, 0.25 percent; citric acid, 0.05 percent; $(NH_4)_2HPO_4$, 0.4 percent; Na_2SO_4, 0.2 percent; KCl, 0.08 percent; Mg, 50 ppm.; Ca (as nitrate), 50 ppm.; Mn, 15 ppm.; Fe, 5 ppm.; Zn, 5 ppm.; and Co, 2 ppm. The medium is adjusted to pH 7.0 and sterilized at 15 pounds for 45 minutes in the fermentor. The pH is maintained within the range 6.7 to 7.0 by periodic additions of NH_4OH during the course of the fermentation. An additional 5 percent of sucrose (50 percent sterile solution) is added incrementally beginning at about the eighth hour of fermentation.

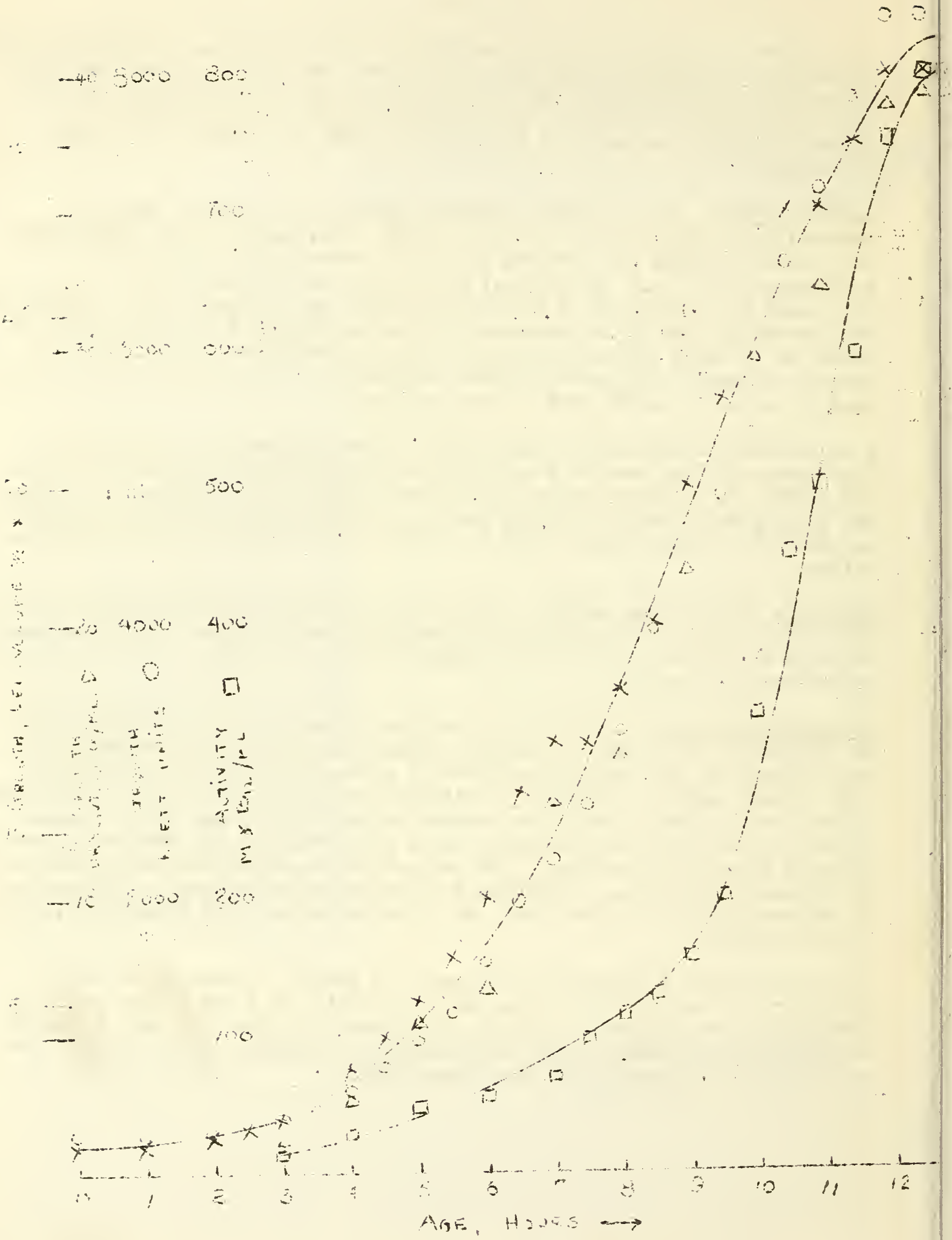
A preliminary experiment was made with a medium containing the following: beet molasses, 12.5 percent (equivalent to approximately 6 percent sucrose); $(NH_4)_2HPO_4$, 0.2 percent; Na_2SO_4, 0.2 percent; KCl, 0.08 percent; Mg, 50 ppm.; Mn, 50 ppm; Zn, 5 ppm; Fe, 5 ppm; and Co, 5 ppm. No yeast extract or citrate was used and no more carbohydrate was added during the course of the fermentation.

Inoculum: Shake flask culture grown 2 days at 25°C. and aerated culture grown overnight at 35°C. have been used alternatively as inoculum. The volume used has been 10 percent. Both citrate-buffered synthetic medium and beet molasses medium have been used.

Fermentation Course: The course of the fermentation on the synthetic medium is illustrated in Figure 1. Growth, whether measured by moist centrifuged cell volume, by dry weight of centrifuged cells, or by bacterial turbidity, gave similar curves. Vitamin B_{12} activity developed somewhat more slowly than growth, but maximum activity was reached within 1 to 2 hours after maximum growth was obtained. The leveling off of the growth and the vitamin B_{12} curves after 12 hours is coincidental with exhaustion of carbohydrate and cessation of ammonia utilization. Continuation of aeration for as long as 10 hours more is accompanied by no marked change in amount of cells or of vitamin B_{12}, and no rise in pH.

In the experiment illustrated in Figure 1, 5 percent of sucrose was added initially and an additional 5 percent was supplied in 5 equal increments at 45-minute intervals between 8.5 and 11.5 hours of growth. If only 5 percent of sucrose is supplied, growth and vitamin B_{12} production level off at about half the levels indicated for this experiment.

Fig. 1 Growth and Vitamin B_{12} Production by B. Megatherium



Cell volume was determined by centrifuging an aliquot of culture at 800 G for 20 minutes in a 10-ml. Kolmer centrifuge tube, and reading the packed cake in percentage of the original culture aliquot. Dry weight was determined on the cell cake, which had been resuspended in water and recentrifuged twice. Bacterial turbidity was measured in the Klett-Summerson colorimeter on a suitable dilution of the culture, and expressed as the product of the arbitrary scale units and the dilution factor.

Poor fermentation results may be obtained if essential mineral elements are precipitated during sterilization. Although substantial citrate concentrations are useful for retaining metals in solution in laboratory investigations, the controlled addition of salts after sterilization and during fermentation should permit the elimination of citrate under larger-scale production conditions. Yeast extract seemed to give some advantage in reducing the lag period but presumably the use of more vigorous inoculum will accomplish the same end.

In preliminary experiments with the beet molasses medium (6 percent sucrose) vitamin B_{12} activity leveled off at 0.44 ppm. at 11 hours. This level is similar to that obtained on synthetic medium with a similar amount of sugar. Attempts to obtain higher levels by incremental feeding and evaluation of other molasses as cheap sources of sugar are in progress. In an experiment with the beet molasses medium, fermentor temperatures of 30° and 37°C. were compared. Growth was about 1 hour slower with the lower temperature, but the same maximum level of B_{12} activity was obtained in either case. Similarly, in an experiment to test the effect of pH, vitamin B_{12} production was only slightly inferior, if at all, when the pH was held in the range of 5.8 to 6.2 than when the pH was held in the range of 6.5 to 7.0.

The vitamin B_{12} activity is entirely associated with the bacterial cells throughout the course of the fermentation. On steaming for 20 minutes all the activity is found in the supernatant. This organism may thus be useful if, on the one hand, it is desired to reduce drying cost by preliminary centrifugation and also if, on the other, it is desired to have a soluble form of vitamin B_{12} for isolation purposes.

A number of cultural and nutritional variables have been investigated. In shake flasks experiments doubling and halving the concentrations of the following inorganic elements were without marked effect on growth (3 and 5 days) or vitamin B_{12} assays (5 days): Ca, K, Co, Mg, Mn, Fe, and Zn. Complete omission of K or Mg reduced growth (and vitamin B_{12}) to a trace. Omission of Ca, Mn, or Fe reduced both growth and vitamin B_{12} to one-fourth to one-half the control level. Omission of Zn or Co was without effect on growth but reduced vitamin B12 to two-thirds for Zn and to one-tenth to one-fourth for Co. Raising the Co level to 4 and 10 times the regular level had no effect. Glucose and maltose proved equally as effective as sucrose for both growth and vitamin B_{12} production. Lactose and galactose did not support growth or vitamin B_{12} production. Addition of 0.2 and 0.4 percent of NaCl had no effect. The shake flask experiments thus indicate that vitamin B_{12} production has more critical nutritional requirements than growth in several respects. Evaluation of these and other factors in aerated vat fermentations is in progress.

Evaluation of B_{12} Potency

L. leichmannii (ATCC 4797) was used for the microbiological assay of vitamin B_{12} as recommended by Skeggs *et al.* (Jour. Biol. Chem. 176, 1459, 1948). The medium was modified in a very important respect by the inclusion of 0.1 percent ascorbic acid as recommended by Stokstad *et al.* (Fed. Proc. 8, 257, 1949), who indicate the function of ascorbic acid to be prevention of destruction of vitamin B_{12} during autoclaving of the assay tubes. In our experience the addition of ascorbic acid together with the use of narrow (15 mm.) culture tubes and adjustment of the basal medium to pH 5.7 yield much more sensitive standard curves and much more consistent assays of microbial cultures. The medium of Skeggs *et al.* was further modified for convenience by the substitutio of a commercial tryptic-digested casein (N-Z-Amine) for that prepared in the laboratory. N-Z-Amine solution is freed of vitamin B_{12} by successive treatment with charcoal and by steaming at pH 1.

All assays are calculated in terms of crystalline vitamin B_{12}, a standardized solution of which was available through the courtesy of Dr. J. C. Woodruff of Merck & Co.

Culture samples for assay purposes were held in the frozen state. Variations such as steaming or autoclaving at pH 7, with or without added ascorbic acid, did not markedly affect the assay values.

Harvested cultures and culture concentrates have been stored under refrigeration without preliminary sterilization, pending chick bioassays. No large losses in activity have been noted. It is believed preferable that such materials be autoclaved if they are to be stored at room temperature. Storage tests are in progress.

The *L. leichmannii* activity produced by *B. megatherium* (B-938) was demonstrate not to be due to thymidine by the instability of the activity on heating in dilute alkali and by the low R_F value found for the active factor on paper chromatography with aqueous *n*-butanol. Culture aliquots of *B. megatherium* were made 0.2*N* with NaOH and steamed for 30 minutes. The samples were then neutralized and assayed microbiologically. Less than 1 percent of the original activity was retained. Thymidine is unaffected by this treatment, according to Dr. B. L. Hutchings. A high-yielding *B. megatherium* (B-938) culture was autoclaved and chromatographed on filter paper with aqueous *n*-butanol. Because of difficulties in detection of vitamin B_{12} activity by the agar plate technique, the chromatogram was cut into narrow strips (R_F increment = 0.05) perpendicular to the direction of solvent flow. Each strip was then assayed with *L. leichmannii* according to the technique outlined by Yacowitz *et al.* (Proc. Soc. Expt. Biol. Med. 71, 372, 1949). Essentially all of the activity was found before $R_F = 0.10$, with the mean R_F approximately 0.04. Crystalline vitamin B_{12} (Merck) behaved similarly.

Chick tests described elsewhere by McGinnis _et al_. (116th National meeting of the American Chemical Society, Atlantic City, September 1949) showed that _B. megatherium_ (B-938) (assaying 0.20 ppm. of vitamin B_{12}), concentrated to 10 times the original culture volume, supplied the requirement of chicks on a vitamin B_{12}-deficient soybean meal ration, at 0.4 percent of the sirup in the ration. Since the sirup assayed 2.0 ppm. of vitamin B_{12} by _L. leichmannii_, this corresponded to a supplementation of the ration with 8 parts per billion of vitamin B_{12}. Chick tests with cultures assaying 0.8 ppm. of vitamin B_{12} are in progress.

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